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Review

Usage of stem cells, neurokines and biomaterials for the repair of peripheral nerve injury

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ABSTRACT

About three percent of trauma patients have peripheral nerve injuries (PNIs), which lead to the loss of motor function. Autologous nerve grafts are still considered as the gold standard for injured peripheral nerve repair, but this would lead to loss of function of the donor sites. Hence, several therapies are being developed for PNI repair of which the three major strategies are: (1) bridging the lesion with biocompatible conduits, (2) usage of stem cells to replace the damaged cells and provide a suitable environment and paracrine factors for nerve regeneration, and (3) usage of neurokines and growth factors to promote nerve regeneration. Combinations of these strategies show better functional recovery in preclinical studies and are likely to become mainstream treatments in the future. In this review, we discuss progress in our work and future direction in using biomaterials, stem cells and neurokines to repair PNIs.

KEYWORDS: peripheral nerve injury, stem cells, induced neurons, nerve conduits, FGF1

Peripheral nerve injury (PNI)

Approximately 2.8% of trauma patients suffer from peripheral nerve injuries (PNIs), which lead to life-long loss of motor function and poor quality of life [1]. According to severity, PNIs are classified into three types: neurapraxia, axonotmesis, and neurotmesis [2]. Neurapraxia, in which the nerve still maintains its continuity and functions, is the mildest PNI. In axonotmesis, the axons and myelin of the nerve at the injury site are lost, but the surrounding connecting tissue such as perineurium and epineurium are preserved. The chances of axon regeneration in axonotmesis is good, because the preserved connective tissue can provide paths for the axons to regenerate into their target tissue such as muscles or sensory organs, and the remaining Schwann cells can secrete neurokines to promote axon regeneration [3]. Neurotmesis means the nerve has been cut. In others words, the injured nerve is discontinuous and has a gap because of which the axons and surrounding connective tissue are disrupted. Without surgical intervention, scar tissue will form at the proximal stump of injured nerve and impede nerve regeneration, and hence functional recovery may not be possible. Before we discuss the developing therapies for PNI repair, we have to understand the mechanism of peripheral nerve regeneration. After axonotmesis and neurotmesis occur, the distal segment of injured nerve undergoes a series of degeneration processes, termed 'Wallerian degeneration'. The axons and the surrounding myelin of distal nerve segment are degenerated at an early stage. The perineurium cells facilitate the infiltration

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of macrophages, and the Schwann cells collaborate with infiltrating macrophages to clear axonal and myelin debris [4]. The disrupted contact with axons triggers the Schwann cells to reach a 'proliferating state' [5], and the proliferating Schwann cells align with each other within endoneurial tubes to form a tube-like structure called band of Büngner which acts as a guide for sprouting axons [6-8]. The Schwann cells within the band of Büngner are shown to express several kinds of cell adhesion molecules and neurokines such as fibroblast growth factor 1 (FGF1), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF), to promote axon regeneration [9-14]. In other words, Schwann cells provide a suitable niche environment for axons to regenerate into their end targets.

In neurotmesis, the whole nerve tissue is lost and scars are formed at both the stumps. Hence, among the three types of PNIs, the repair of neurotmesis is the most challenging. In clinics, when the gap between two disconnected nerve stumps is too large to suture, it must be bridged by using autologous nerve grafts to prevent the formation of scar tissue and to provide a suitable environment for axon sprouting. Although autologous nerve grafts is considered the 'gold standard' in PNI repair, this would lead to loss of function of the donor sites. The size of autologous nerve might be insufficient for the PNI repair, and choices of donor nerve are limited, especially in patients with chronic diseases, such as diabetes. Furthermore, only 40-50% of PNI patients cured via autologous nerve grafts show function recovery [15]. As a result, several therapies were developed for PNI repair: (1) usage of biocompatible conduits to bridge the lesions; (2) usage of stem cells to replace the damaged cells and provide suitable environments for nerve regeneration; and (3) usage of neurokines and growth factors to promote nerve regeneration. Combinations of these strategies show better functional recovery in the preclinical studies, and are likely to become mainstream treatments in the future. Here, we discuss the recent progress in the usage of biomaterials, stem cells and neurokines to repair PNIs.

Isolation of neural stem cells (NSCs)

Neural stem cells are capable of self-renewal and differentiation into three neural lineages, namely neurons, astrocytes, and oligodendrocytes. Therefore, NSCs are the best cellular sources for the treatment of neurological diseases, including PNIs [16]. However, an important issue in the NSC-based therapies is how to obtain enough cells for treatment. To resolve this problem, one must isolate NSCs with excellent cell-renewal and proliferation ability. Flow cytometry and fluorescence-activated cell sorting (FACS) have been applied extensively to isolate different populations of stem cells. Using FACS, NSCs can be isolated from fetal and adult brain by surface markers, such as CD133 or fluorescent proteins driven by the NSC-specific promoters, including Sox2, Nestin, and FGF1 [17-19].

FGF1 is expressed predominantly in the nervous system, including the brain and retina [20-22], and has multiple functions in the nervous system. FGF1 has been shown to have neurotrophic effect on primary peripheral and central nervous system neurons. For example, FGF1 can promote the mitogenesis in glial cells [23, 24] and neuroblasts [25], and enhance the neurite outgrowth in ganglion cells [26] and PC12 cells [27]. Furthermore, FGF1 plays a role in sustaining the proliferation and self-renewal of NSCs [28, 29]. Human FGF1 has four alternative splicing forms, designated 1A, 1B, 1C, and 1D, which are different in the upstream un-translated exon, but code for the same polypeptide [30, 31]. These four alternative splicing forms of human FGF1 are expressed in different tissues via the control of different promoters [30, 32]. The FGF1 transcript that is expressed predominantly in human brain and retina is FGF1B [30, 33]. In addition, mouse FGF1B transcript is expressed in the areas that are shown to be abundant with NSCs [34]. By using luciferase reporter assay, we have identified human FGF1 promoter 1B, designated F1B [32, 35]. To investigate the expression of human F1B promoter, we generated transgenic mouse in which SV40 T antigen (Taq) is driven by human F1B promoter, and found that human F1B promoter is active in the NSC-rich regions, such as subventricular zone [29]. This suggests that the cells in which F1B promoter is active are NSCs and that we could use F1B as a selection tool to isolate NSCs. Thus, the F1B-GFP reporter in which GFP expression is under the control of human F1B promoter was generated and used to isolate NSCs from adult and fetal mouse brain. We found that the F1B-GFP positive cells generate more neurospheres

than the F1B-GFP negative cells (Figure 1), and can differentiate into neurons, astrocytes, and oligodendrocytes [19, 36]. This indicates that the NSCs isolated via F1B-GFP reporter can self-renew and are multipotent, and might be promising for the treatment of neurological disorders. The F1B-GFP positive NSCs have been shown to facilitate the sciatic nerve regeneration in rats (see below). We speculate that the F1B-GFP positive NSCs could also be used to treat neurodegenerative diseases, such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD). In order to improve the performance of the NSC-based cell therapies in neurological disorders, we developed a novel biomaterial called ultra-nanocrystalline diamond (UNCD), which can promote the differentiation of NSCs (Figure 2). In addition, different modifications on the UNCD surface can promote the differentiation of NSCs toward different neural lineages [37, 38]. Therefore, UNCD could be used to control and enhance NSC differentiation in the future.

Generation of induced neural stem cells (iNSCs) and induced neurons (iNs) by cellular reprogramming

Human NSCs must be isolated from fetal brain and hence the sources of human NSCs are limited and the usage of human NSCs is abound with ethical controversy. Dr. Shinya Yamanaka generated induced pluripotent stem cells (iPSCs) from human and mouse skin-derived fibroblasts by using four reprogramming factors namely *OCT4*, *SOX2*, *KLF4*, and *c-MYC* [39, 40], and established a new field of cell reprogramming for which he received the Nobel Prize in 2012. The iPSCs generated from adult skin-derived specimens avoided ethical issues. However, the differentiation of iPSCs is hard to control, and the transplantation of iPSCs usually leads to tumor formation [41]. Direct reprogramming is a novel reprogramming technique of directly converting cells into various cell types, bypassing the pluripotent state by using lineage-specific transcriptional factors. Hence, various cell types which are without the risk of tumor formation

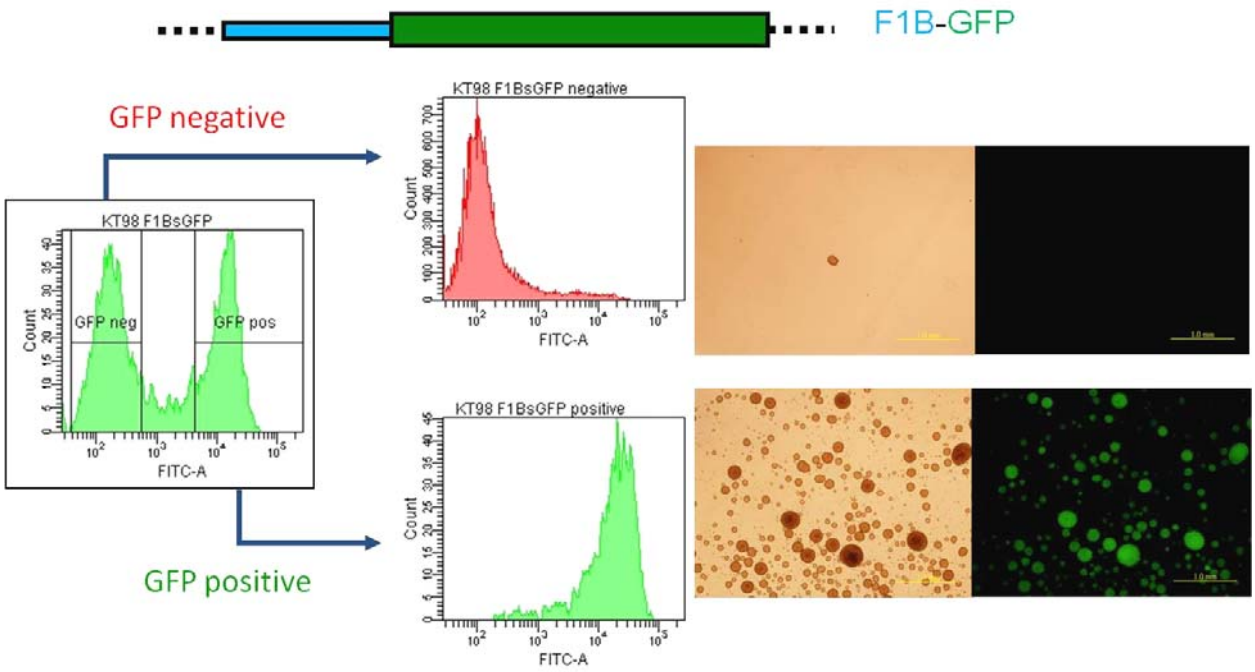

Figure 1. NSCs isolated via F1B-GFP reporter can self-renew, and are with higher proliferation capability. F1B-GFP reporter comprises the coding sequence of GFP driven by the brain-specific promoter of FGF1 (F1B). Using FACS, mouse brain cells are sorted into two populations: F1B-GFP positive and F1B-GFP negative. F1B-GFP positive cells form more neurospheres than F1B-GFP negative cells. Thus, cells isolated via F1B-GFP reporter selection possess the properties of NSCs.

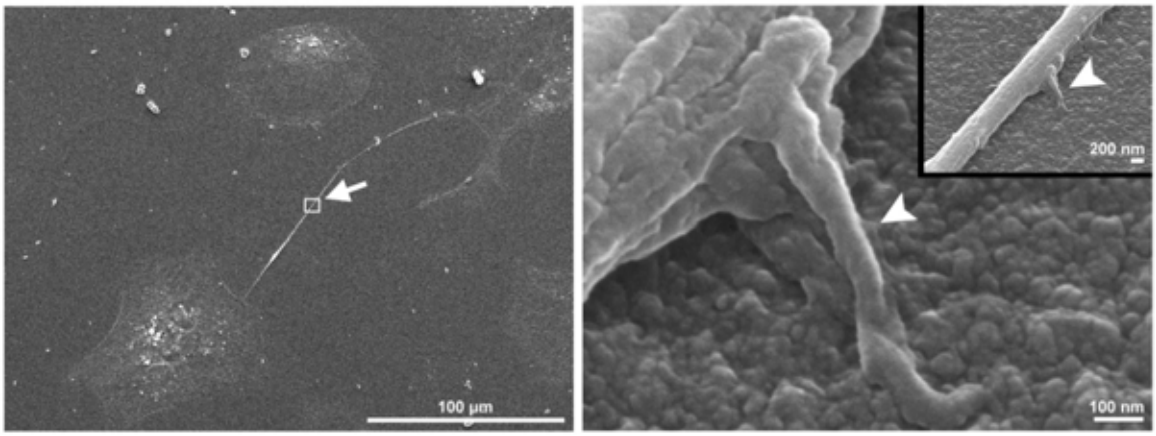

Figure 2. Scanning electron microscope images of NSCs cultured on UNCD films. UNCD films can promote the differentiation activity of NSCs without any inducing reagents. The photograph in the right is an enlarged version of the white box indicated by an arrow in the photograph in the left. Arrowheads indicate the filopodia that was adhered to the UNCD film.

can be generated for cell therapies. Kim *et al*. first generated induced neural stem cells (iNSCs) from mouse embryonic fibroblasts (MEF) by temporal expression of *OCT4*, *KFL4*, *SOX2*, and *c-MYC*. They also demonstrated that iNSCs were directly induced from MEF but not from pluripotent intermediates. iNSCs have similar gene expression profile with NSCs, can differentiate into the three neural lineages, and have self-renewal capacity [42]. Several combinations of transcriptional factors were identified to be sufficient for reprogramming somatic cells into iNSCs [43-48]. Furthermore, Pei *et al*. found that MEF and human urinary cells could be converted into iNSCs by using chemical cocktails (VPA, CHIR99021, and Repsox) under a hypoxia culture condition [49]. Taken together, iNSCs could be generated safely and ethically for the treatment of neurological diseases.

Although NSCs can differentiate into neural cells, the efficiency of neuron differentiation is low. Therefore, induced neurons (iNs) that are directly induced from somatic cells might be more promising than iNSCs for the treatment of neurological diseases. The first iNs were induced from fibroblasts by expression of the neural transcription factors such as *ASCL1*, *BRN2*, and *MYT1L* [50]. Other neural transcription factors such as *miR124*, *MYTL1*, and *BRN2* (IBM) were also shown to be able to convert somatic cells into iNs. The iNs induced by IBM show neuronal morphology and marker expression, could fire action potential, and form synapses [51]. SH2B1 is an adaptor/scaffold protein and it has three proline-rich domains, a pleckstrin homology domain, and a C-terminal Src domain. In our previous studies, we have shown that SH2B1 could enhance the neurite outgrowth induced by FGF1 through FGFR1-Mek-Erk1/2-Stat3- Egr1 signaling pathway [52, 53]. In addition, SH2B1 could also enhance the NGF and glia-derived growth factor (GDNF)-mediated neurite outgrowth [54-58]. Therefore, we combined SH2B1 with IBM (S-IBM) to reprogram human fibroblasts into iNs (Figure 3). We found that S-IBM could speed up the maturation of iNs, and also increase the length and number of neurite outgrowth [59]. S-IBM could generate iNs more efficiently, and this will facilitate the application of iNs in the treatment of neurological diseases. Thus, we can generate patient-specific iNSCs and iNs from somatic cells to treat neurological diseases, and avoid the risk of tumorigenesis.

Usage of stem cells, neurokines and biomaterials for the repair of peripheral nerve injuries

Artificial nerve graft is a synthetic nerve conduit which bridges the two stumps of nerve gap, prevents the formation of scar tissue, and facilitates nerve regeneration. The material that was used first in PNI repair is silicon. Although silicon is biocompatible, it is not biodegradable, and leads to some long-term complications such as nerve compression and

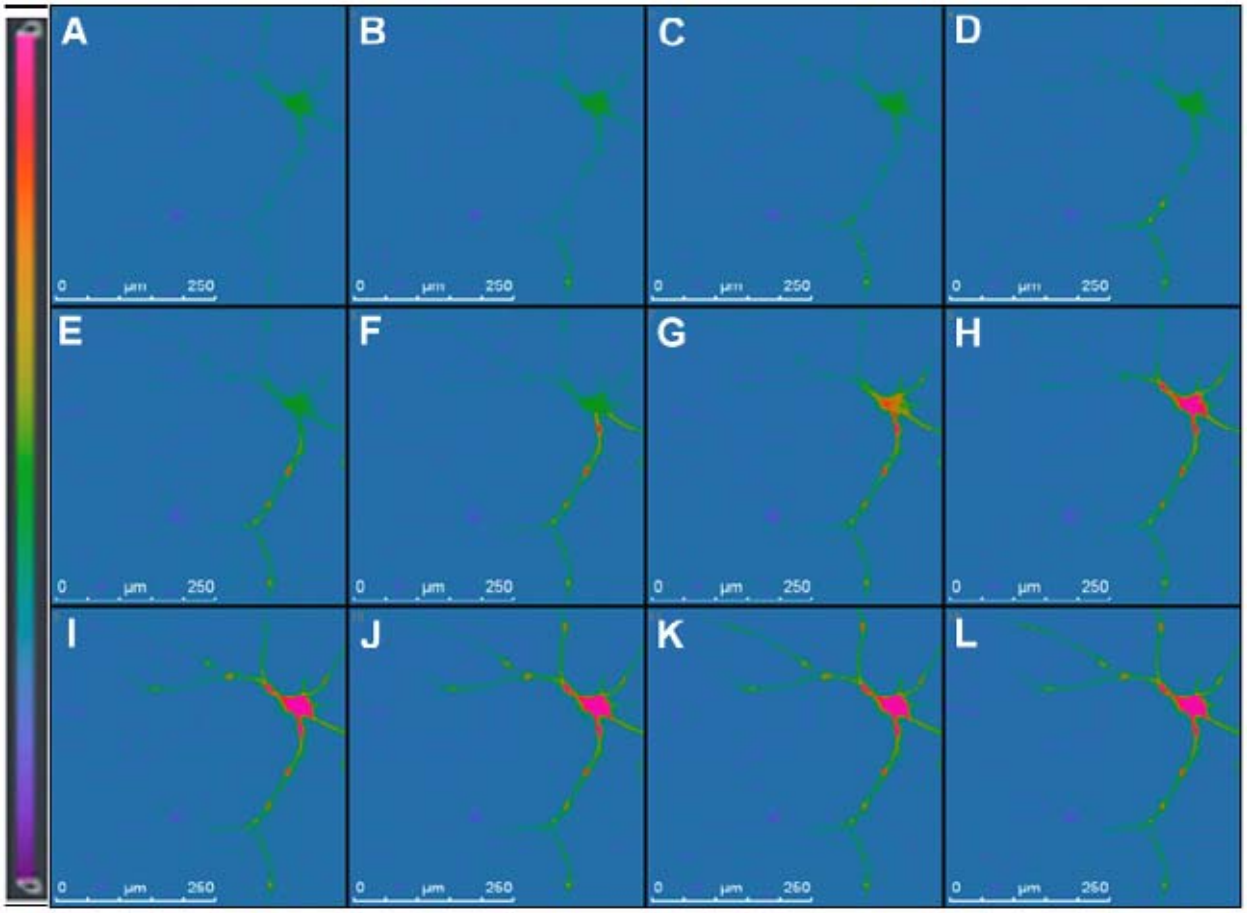

Figure 3. After glutamate stimulation, iNs induced by *SH2B1*, *miR124*, *MYTL1*, and *BRN2* (S-IBM) show calcium influx activity. INs induced by S-IBM were labeled with Fura2-AM, which is an intracellular calcium indicator. After glutamate stimulation, iNs induced by S-IBM show calcium influx activity. It indicates that iNs induced by S-IBM behave as functional neurons.

fibrosis. Hence, a second surgery is required for the removal of silicon conduit [60]. To avoid the complications from non-resorbable conduits, a variety of biodegradable materials were tested for PNI repair. Polylactic acid (PLA), which is a kind of polyester derived from renewable resources, such as tapioca roots and corn starch, is both biodegradable and biocompatible. PLA is easily fabricated and does not induce inflammatory response [61]. Hence it is suitable for the production of biodegradable nerve conduits. The PLA conduits with an asymmetrical structure have high outflow permeability and can promote the nerve regeneration of rats with PNIs [62, 63]. Although artificial nerve conduits provide a promising alternative to replace autologous nerve grafts, the efficacy of nerve conduits is not as good as autologous nerve grafts. This is because the Schwann cells within autologous nerve grafts can express several kinds of cell adhesion molecules and neurokines to facilitate the axon regeneration. Therefore, the combinations of nerve conduits and neurokines/stem cells might be more efficient. It implies that using the PLA conduits along with the F1B-GFP positive NSCs could repair PNIs more efficiently. In order to align the F1B-GFP positive NSCs on PLA conduits, we fabricated micro-patterned PLA conduits having micro-patterned structure [64]. We found that the F1B-GFP positive NSCs can attach and proliferate on micro-patterned PLA conduits and express more NGF and BDNF than the F1B-GFP negative NSCs on non-patterned PLA conduits. As anticipated, micro-patterned PLA conduits along with the

F1B-GFP positive NSCs show better functional recovery and more myelinated axons in rats with PNIs than conduits alone [64]. This indicates that the combination of PLA conduits and F1B-GFP positive NSCs repair PNIs more efficiently, probably due to the similarity to autologous nerve grafts. As mentioned above, FGF1 functions as a neurokine in neurons of peripheral and central nervous system. FGF1 could facilitate the motor function recovery of rats with spinal cord injury [65-67], and has been used to treat the spinal cord injury of human in clinical studies [68]. Artificial nerve conduits along with FGF1 showed more regenerating axons and better functional recovery than conduits alone in PNI repair of rats [69, 70]. We fabricated PLA/FGF1 conduits in which FGF1 are immobilized by chitosan-Au grafting, and found that FGF1 are released slowly in these conduits [71]. The PNI rats repaired by PLA/FGF1 conduits showed better motor function recovery and more regenerating axons than those repaired by PLA conduits. Furthermore, PLA/FGF1 conduits were seeded with F1B-GFP positive NSCs and were used to repair the 1.5 cm nerve gap of rats. We found that the PNI rats repaired by PLA conduits containing FGF1 and F1B-GFP positive NSCs showed excellent function recovery (Figure 4), comparable to those repaired by autologous nerve grafts [71]. This indicates that the usage of NSCs, FGF1, and PLA conduits is a promising alternative for autologous nerve grafts in the repair of PNIs.

Future perspective

Although we can generate iNSCs/iNs safely and ethically from somatic cells, we still have to test if the performance of iNSCs/iNs in PNI repair is as good as NSCs. We intend to test if iNSCs/iNs could repair PNIs, and if F1B-GFP could facilitate the isolation of iNSCs/iNs. Besides Schwann cells, inflammatory cells, such as macrophages, are also involved in PNI repair. This implies that not only neurokines but also inflammatory cytokines might participate in the repair of PNIs. We are in the process of identifying inflammatory cytokines that might facilitate PNI repair. We successfully repaired the PNIs of rodents by using nerve conduits, stem cells and neurokines. It might be a promising alternative for autologous nerve grafts in PNI repair.

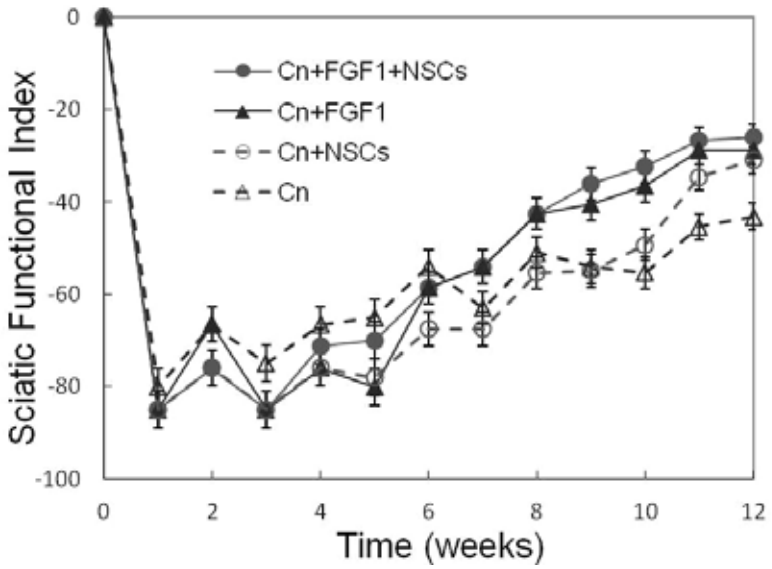

Figure 4. Sciatic functional index of PNI rats repaired by PLA conduits with FGF1 and NSCs. Cn: PNI rats repaired using conduits alone (Δ); Cn+NSCs: PNI rats repaired using conduits with NSCs (O); Cn+FGF1: PNI rats repaired using conduits with FGF1 (▲); Cn+FGF1+NSCs: PNI rats repaired using conduits with FGF1 and NSCs (●). Cn+FGF1+NSC group shows better functional recovery than the other groups. This indicates that using stem cells, neurokine, and conduits is the best strategy for PNI repair.

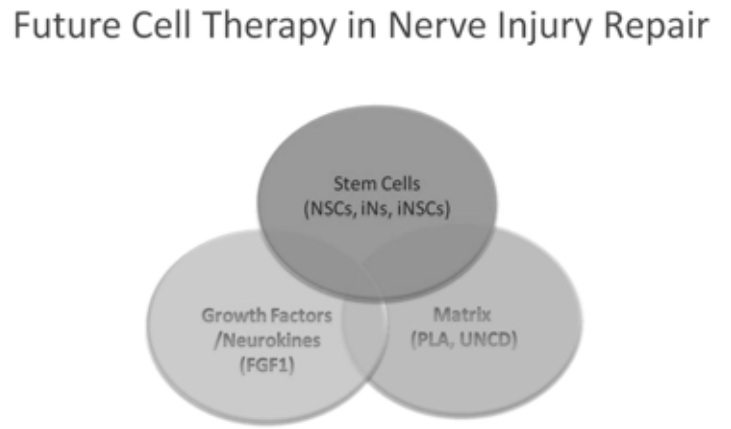

Figure 5. Using stem cells, neurokines and biomaterials to repair peripheral nerve injury is likely to become mainstream treatments in the future.

However, PNI repair in rodents is faster and simpler than those in humans because rodents have a faster nerve regeneration rate and smaller body size [72]. Hence, there is a need for setting up a large animal model of PNI repair. The mini-pig is more similar to humans in anatomy and physiology, and we intend to set up a model of PNI repair in mini-pig. It is anticipated that the successful execution of PNI repair in large animals will help in paving the way for clinical trials. Taken together, the usage of stem cells, neurokines and biomaterials to repair peripheral nerve injuries is likely to become mainstream treatments in the future (Figure 5).

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest related to this article.

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